IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S)	: BOWEN, Philip J. et al
SERIAL NO.	: 10/502,080
FILED	: October 8, 2004
FOR	: SOLENOPSIN A, B AND ANALOGS AS NOVEL ANGIOGENESIS INHIBITORS
GROUP ART UNIT	: 1612
Examiner	: Brian M. Gulledge

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Commissioner for Patents
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FURTHER DECLARATION OF DR. JACK L. ARBISER

I, Jack L. Arbiser declare as follows:

1. I am a co-inventor of the subject matter of the above-referenced patent application.

2. In 1983, I received a B.S. degree in Chemistry from Emory University, Atlanta, Georgia.

3. In 1991, I received a Ph.D. degree in Genetics and a MD degree in Medicine from Harvard Medical School, Boston, Massachusetts.

5. From 1994-1998, I participated in the Howard Hughes Postdoctoral Fellowship, Laboratory of Judah Folkman, M.D., Harvard Medical School, Boston Massachusetts.

6. Since 1991, I have studied the mechanisms of how oncogenes and tumor suppressor genes regulate angiogenesis and tumorigenesis. This work has resulted in the

discovery of small molecule inhibitors of tumor growth, and a novel method of predicting tumor signaling based upon loss of tumor suppressor genes p53 and p19ink4a. I have substantial scientific and medical expertise in cancer and clinical oncology.

7. I am presently a Professor of Dermatology, Emory University School of Medicine, Atlanta, Georgia. I have held this position since September, 2009.

8. I am also presently an Attending Physician, Atlanta Veterans Administration Medical Center, Atlanta Georgia. I have held this position since 2001.

9. In 2008, I was the Chief of Service of Dermatology and the Chief of Dermatology, Atlanta Veterans Administration Medical Center, Atlanta Georgia.

10. In 2007, I was the Director of Research, Department of Dermatology, Emory University School of Medicine, Atlanta, Georgia.

11. From 2004 to 2009, I was an Associate Professor in the Department of Dermatology, Emory University School of Medicine, Atlanta, Georgia.

12. From 1998 to 2004, I was an Assistant Professor in the Department of Dermatology, Emory University School of Medicine, Atlanta, Georgia.

13. In 1998, I was an Attending Physician, Emory University School of Medicine, Atlanta, Georgia

14. From 1995 to 1998, I was an Instructor in the Department of Dermatology, Harvard Medical School, Boston, Massachusetts.

15. From 1992 to 1994, I was a Resident in Dermatology, Massachusetts General

Hospital, Boston, Massachusetts.

16. From 1991 to 1992, I was an Intern in Internal Medicine, Beth Israel Hospital, Boston, Massachusetts.

17. From 1985 to 1991, I was in the Medical Scientist Training Program, Department of Genetics, Harvard Medical School, Boston, Massachusetts.

18. In 1984, I was a Research Assistant, Department of Rheumatology, Massachusetts General Hospital, Boston, Massachusetts.

19. In 1983, I was a Research Assistant, Department of Pediatrics, Emory University, Atlanta, Georgia.

20. In 1979, I was an Undergraduate Research Assistant, Department of Chemistry, Emory University, Atlanta, Georgia.

21. I have received numerous awards and honors for my scientific work including receiving the Albert E. Levy Scientific Research Award for Senior Investigator in 2007, and receiving the Emory School of Medicine Dean's Clinical Scholar award from 2000-2003 and 2004-2006 among other awards and honors.

22. I am a member of the Emory Medical Student Research Committee (2001-present) and the VA Research and Development Committee (2007-present). I am also a member of the Dermatology Foundation Metical and Scientific Committee External Advisory Board, University of Arizona Cancer Center, the Sturge-Weber Foundation Scientific Advisory Board (2001-present) and the American Academy of Dermatology-NAID Liaison (1998-present). I was an Organizer for the 48th Montagna Annual Symposium on the Biology of Skin, Snowmass Colorado (1999). I have been a Membership Chair of the Society for Investigative Dermatology

(2001-2002) and a Resident/Fellow Representative for the Society of Investigative Dermatology (1995-1997).

23. I have been a member of the following societies: the American Association for Cancer Research, The Society for Investigative Dermatology, the American Academy of Dermatology, the Tuberous Sclerosis Alliance, the Dermatology Foundation and the Sturge-Weber Foundation.

24. I am on the Editorial Boards for Pigment Cell Research (2007-present) and Journal of Investigative Dermatology (2001-present). I have been on the Editorial Boards for Journal of the Cutaneous Medicine and Surgery (2002-2005) and Journal of the American Academy of Dermatology (2001-2004). I was also a Guest Editor for Seminars in Cancer Biology, Karolinska Institute.

25. I have published over 200 scientific papers and I have published extensively in the scientific area of cancer research, including the mechanism by which cancer occurs, including the role of angiogenesis in cancer pathogenesis, including tumorigenesis.

26. The above paragraphs clearly establish a foundation for my medical and scientific expertise in cancer, including experimental cancer and clinical oncology, in providing the instant declaration.

27. I am familiar with United States patent application serial number 10/502,080, of which I am a co-inventor. I understand that the presently pending claims are directed to a method of treating cancer or a tumor in a patient comprising administering to a patient in need an effective amount of a composition which comprises a compound as otherwise set forth in the presently pending claims, namely claims 40, 50-56 and 66. Essentially, the presently pending claims are directed to the discovery that compositions which contain effective amounts of a compound as claimed are effective to treat a number of cancers and tumors. This is based upon

the fact that the compounds which are set forth in presently pending claims 40, 50-56 and 66 inhibit cancer and/or tumor growth by a mechanism which inhibits angiogenesis in the cancer/tumor tissue. By inhibiting angiogenesis, the presently claimed methods provide a generic approach to the treatment of any number of cancers and tumors as set forth in presently pending claims 40, 50-56 and 66.

28. It is my opinion that the presently claimed methods of treating tumors and/or cancer are useful and are expected to work as described and claimed, given that angiogenesis is an important mechanism by the way tumors and/or cancer grow and elaborate, and the presently claimed methods set forth in pending claims 40, 50-56 and 66 are directed to methods which utilize the inhibition of angiogenesis as a general mechanism to treat tumor and/or cancer.

29. Angiogenesis comprises the development of a new vasculature for a tissue with increased metabolic demand. In adult life, the new tissue is likely to be a tumor or cancer, either benign or malignant, or an inflammatory process, such as psoriasis, inflammatory bowel disease, arthritis, asthma, multiple sclerosis, type II diabetes, lupus, and other diseases. The major sources of the blood vessel cells (endothelial cells) that are required for this process are recruitment of blood vessel cells from local pre-existing capillaries, or recruitment of cells from bone marrow that can turn into endothelial cells. Both processes contribute to the vascularization of a tumor or an inflammatory process. One of the commonalities of both inflammatory and tumor derived (neoplastic processes) is that they elaborate factors that recruit endothelial cells. The major factors for these processes include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), inflammatory cytokines (such as interleukin-8, and other factors), which stimulate the migration and proliferation of endothelial cells. The laboratory of Judah Folkman, MD, proved the absolute requirement of angiogenesis for the growth of malignant tumors, obesity, maintenance of pregnancy, and development of atherosclerotic plaques. Based upon this pioneering work, angiogenesis inhibitors have been developed for the treatment of human diseases, in particular the treatment of tumors and cancer.

26. Two strategies have been developed for the assessment of angiogenesis inhibitors. The first is direct inhibition, in which the activity of growth factors on the receptors is directly antagonized. The second is indirect inhibition, in which the ability of tumors to produce growth factors is inhibited. The phosphoinositol-3 kinase pathway, which is activated in virtually all tumors, is implicated in both direct and indirect angiogenesis inhibition.

27. Direct antiangiogenesis inhibition is now in clinical use for the treatment of cancer. The most prominent example is that of avastin (bevacizumab) that directly blocks the activity of VEGF on endothelial cells. Avastin is commonly used for the treatment of kidney and colon cancer, and more recently in brain cancer (glioblastoma). While avastin has been shown to be of clinical benefit, it is not curative and has well known side effects, such as hypertension and bleeding. In addition, the tumor hypoxia that is induced by avastin is thought to cause adaptation in the tumor, such as increased local invasion and elaboration of more growth factors in order to relieve the avastin-induced tumor hypoxia. Other strategies are being developed, but it is likely that any strategy that increased tumor hypoxia by itself will ultimately not cure a tumor.

28. Indirect inhibition of angiogenesis is an attractive strategy that has not been sufficiently explored. My studies in the Folkman lab, published in the Proceedings of the National Academy, were the first to demonstrate that blockade of phosphoinositol-3 kinase was able to inhibit the growth of a tumor *in vivo*. Blockade of phosphoinositol-3 kinase is an attractive strategy for several reasons. First, it blocks the production of growth factors by the tumor. Second, it causes increased apoptosis (programmed cell death) in tumors themselves. Finally, it is believed to prevent the metabolic adaptations in tumors caused by antiangiogenic therapies. Since phosphoinositol-3 kinase is such an important target, we regard it as a major focus to inhibit this pathway and to treat tumors and cancer. We discovered that solenopsin, a naturally occurring alkaloid in the venom of the fire ant (Solenopsis invicta), is a potent inhibitor of this enzyme. In addition, we have shown that this compound claimed in the present invention is a potent inhibitor of angiogenesis in the zebrafish model and that solenopsin is stable and may

be used as a pharmaceutical agent. See the attached references, Arbiser, et al, *Blood*, 15 January 2007, Volume 109, Number 2, pages 560-565, which teaches the inhibition of angiogenesis by solenopsin consistent with its use as an anticancer agent, and Park, et al., *Journal of Infectious Diseases*, 15 October 2008, 198, 1198-201, which teaches that solenopsin is stable and may be used as a small molecule pharmaceutical agent as set forth in the present invention. The inhibitory activity, small molecular size and stability of solenopsin, which make it amenable to topical, systemic and oral administration, make it an attractive molecule for the treatment of tumors and cancer. It thus represents close to an ideal compound for providing generic therapy against a variety of cancerous tissue.

29. By way of cellular experimental evidence, recently the following experiments on a benign tumor cell line (FP52-SV) and malignant sarcoma cell (tsc2ang1) were conducted. In these experiments, the anti-proliferative/anticancer activity of solenopsin A (a compound claimed in the present application) were tested in the two cell lines. More specifically, 10,000 cells from benign tumors (FP52-SV40) or malignant sarcoma cells (tsc2ang1) were plated in 24 well plates. 24 hours after plating, the cells were treated with solenopsin in concentrations ranging from 0-20 micromolar or vehicle control. A number of other compounds were also tested. 24 hours after solenopsin treatment, the cells were trypsinized and counted with a cell culture.

30. The results of the experiments which are described in paragraph 29, above are shown in the attached Exhibits 1 and 2. In the first assay, the proliferation assay performed on the benign tumor cell line FP52 SV40, the anti-proliferative effect of solenopsin on the cells is clearly evidenced (see attached Exhibit 1). Noted is the fact that a concentration of solenopsin at 10 micromolar provided significant inhibition of cell proliferation (approximately 80%). The graph also evidences that anti-proliferative activity of solenopsin was synergistic with imipramine blue where both compounds were used at a concentration of 1 micromolar. In the second experiment, the effect of solenopsin at varying concentrations was tested against malignant sarcoma TSC2ang1 cells (Exhibit 2). In this experiment, solenopsin exhibited

excellent antiproliferative/anticancer activity against the sarcoma cell line with a concentration of 10 micromolar solenopsin being particularly effective, with concentration of solenopsin of 15 and 20 micromolar being slightly less effective than the lower concentration of 10 micromolar. In both experiments, the results of which are presented in attached Exhibits 1 and 2, solenopsin showed substantial antiproliferative/anticancer activity in cell-based assays consistent with its use as an anticancer agent as claimed in the present invention.

31. Given the exceptional inhibitory activity solenopsin displays against phosphoinositol-3 kinase and the direct and indirect role that phosphoinositol-3 kinase plays in promoting angiogenesis, a critically important process in tumor/cancer growth and elaboration, as well as the experimental test results which are described in paragraphs 29 and 30, above, it is my expectation that solenopsin will prove to be an effective agent against tumors and cancer by inhibiting angiogenesis in cancer tissue through inhibition of phosphoinositol-3 kinase. This expectation is corroborated by the favorable antiproliferative/anticancer activity exhibited by solenopsin in the cell-based assays which are described above and in attached Exhibits 1 and 2. By virtue of the inhibitory activity of the compounds which are presently claimed in the pending method claims, it is my expectation that these compounds will prove to be effective anti-cancer agents against a broad range of tumors and cancer.

32. I further declare that all statements made herein of my own personal knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 2/28/11

Jack L. Abiser, MD, PhD

EXHIBIT 1

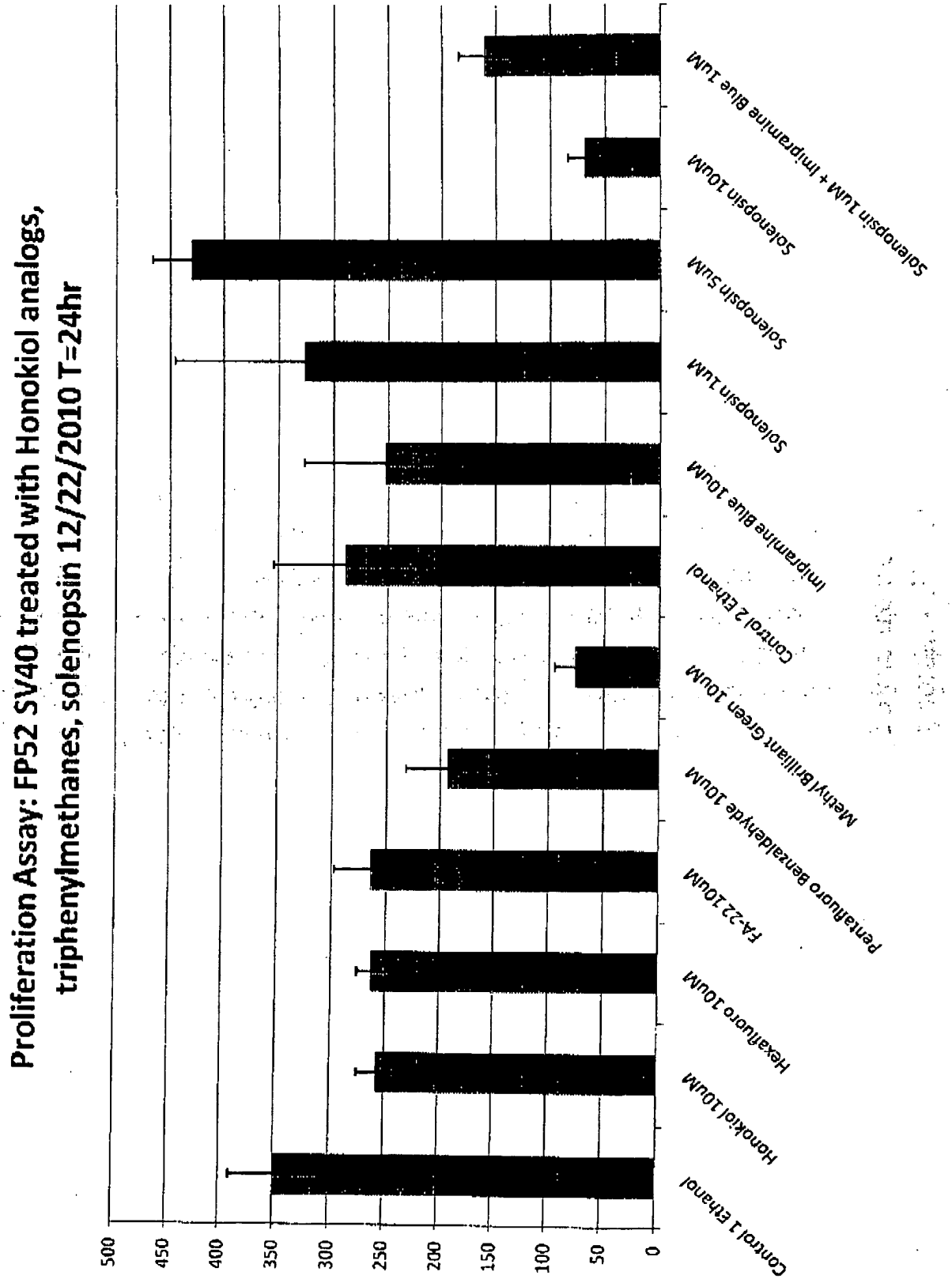
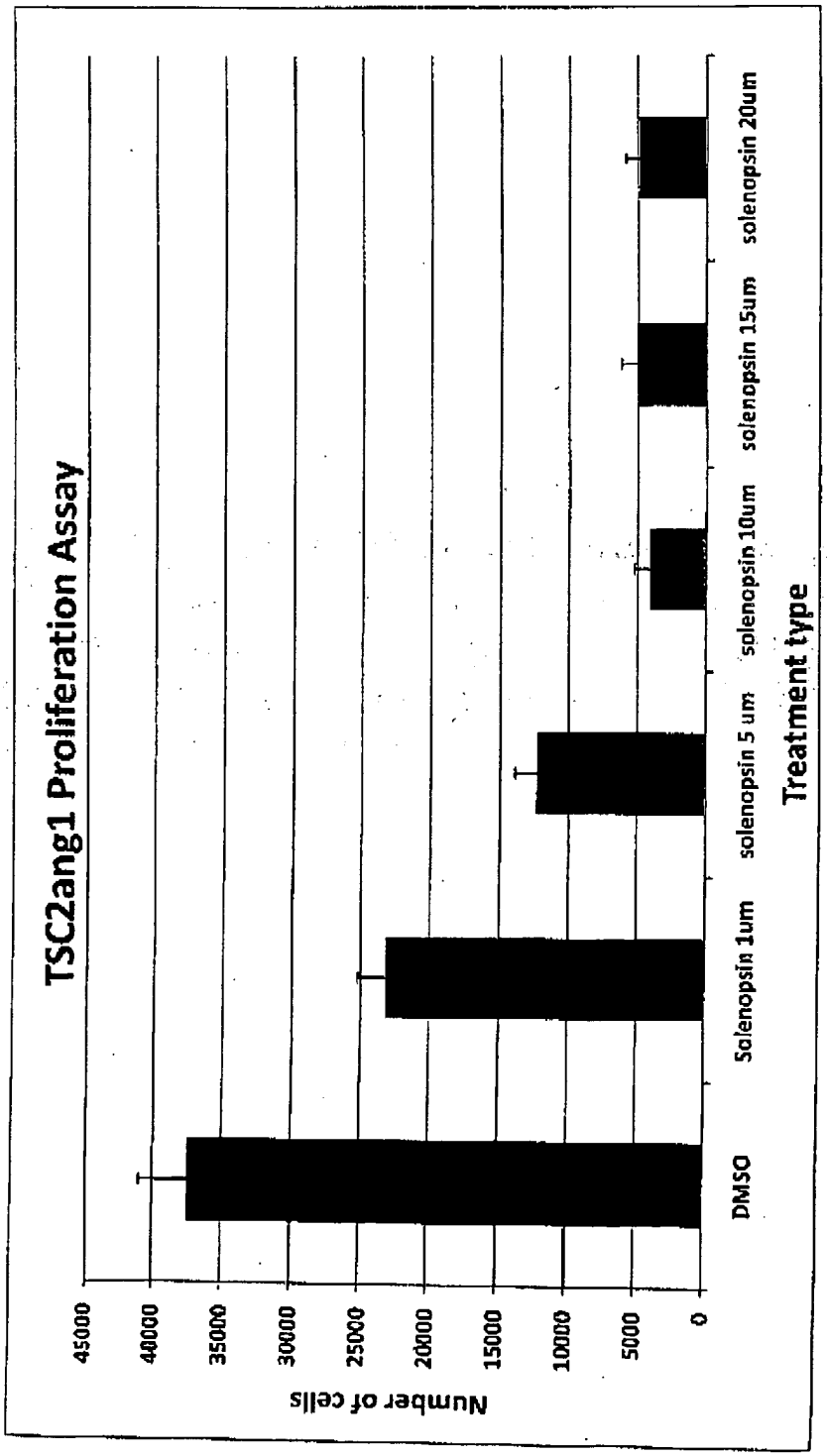

Proliferation Assay: FP52 SV40 treated with Honokiol analogs, triphenylmethanes, solenopsin 12/22/2010 T=24hr
500
450
400
350
300
250
200
150
100
50
0
Control 1 Ethanol
Honokiol 10uM
Hexafluoro 10uM
FA-22 10uM
Pentafluoro Benzaldehyde 10uM
Methyl Brilliant Green 10uM
Control 2 Ethanol
Imipramine Blue 10uM
Solenopsin 1uM
Solenopsin 5uM
Solenopsin 10uM
Solenopsin 1uM + Imipramine Blue 1uM

EXHIBIT 2


TSC2ang1 Proliferation Assay
Number of cells
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
DMSO
Solenopsin 1um
solenopsin 5 um
solenopsin 10um
solenopsin 15um
solenopsin 20um
Treatment type

Solenopsin, the alkaloidal component of the fire ant (*Solenopsis invicta*), is a naturally occurring inhibitor of phosphatidylinositol-3-kinase signaling and angiogenesis

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Phosphatidylinositol-3-kinase (PI3K), and its downstream effector Akt, or protein kinase Bα (PKBα), play a major regulatory role in control of apoptosis, proliferation, and angiogenesis. PI3K and Akt are amplified or overexpressed in a number of malignancies, including sarcomas, ovarian cancer, multiple myeloma, and melanoma. This pathway regulates production of the potent angiogenic factor vascular endothelial growth factor (VEGF), and protects tumor cells against both chemotherapy and reactive oxygen–induced apoptosis through phosphorylation of substrates such as apoptotic peptidase–activating factor-1 (APAF-1), forkhead proteins, and caspase 9. Given its diverse actions, compounds that suppress the PI3K/Akt pathway have potential pharmacologic utility as angiogenesis inhibitors and antineoplastic agents. Using the SVR angiogenesis assay, a screen of natural products, we isolated the alkaloid solenopsin, and found that it is a potent angiogenesis inhibitor. We also found that solenopsin inhibits the PI3K signaling pathway in cells upstream of PI3K, which may underlie its effects on angiogenesis. Consistent with inhibition of the activation of PI3K, solenopsin prevented the phosphorylation of Akt and the phosphorylation of its substrate forkhead box 01a (FOXO1a), a member of the forkhead family of transcription factors. Interestingly, solenopsin also inhibited Akt-1 activity in an ATP-competitive manner in vitro without affecting 27 of 28 other protein kinases tested. (Blood. 2007;109:560-565)

Introduction

The serine/threonine kinase c-Akt-1, or protein kinase Bα (PKB), is the cellular homolog of a transforming oncogene initially isolated from a lymphoma. Akt is a downstream target of phosphatidylinositol-3-kinase (PI3K), a family of at least 4 different enzymes, with the prototypical PI3K heterodimer consisting of a p85 (regulatory) and a p110 (catalytic) subunit. The PI3K/Akt pathway is involved in the regulation of diverse cellular functions including proliferation, cytoskeletal organization, survival, and malignant transformation.[1-4] Upon binding of PI3K products to its pleckstrin homology domain, Akt is translocated to the plasma membrane where it is activated by upstream phosphorylated kinases, including PI3K-dependent kinases 1 and 2 (PDK1 and PDK2) and mammalian target of rapamycin complex 2 (mTORC2). The PI3K/Akt pathway is stimulated by numerous receptor tyrosine kinases and oncogenes, including receptors for insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), ras, Her2/neu, and polyoma middle T oncogenes.[5-10] Because Akt plays a central role in regulating apoptosis, angiogenesis, and metabolism of cells, Akt is an attractive pharmacologic target for the treatment of cancer and inflammation.[11,12] Small-molecular-weight inhibitors of PI3K include LY 294002 and the fungal metabolite wortmannin,[13] as well as ether phospholipids, including perifosine, which has entered clinical trials.[14,15]

Using the SVR angiogenesis assay,[16-18] we found that solenopsin A,[19,20] the primary alkaloid from the fire ant *Solenopsis invicta*, has antiangiogenic activity. We also discovered that solenopsin disrupted angiogenesis in vivo in embryonic zebrafish. In order to determine the mode of action, we examined the ability of solenopsin to inhibit a battery of cellular kinases and found that solenopsin A inhibited Akt relatively selectively in an ATP-competitive manner without affecting its upstream activator PDK1 or PI3K. However, in cells, solenopsin prevented the activation of PI3K, the phosphorylation of Akt-1 at both Thr308 and Ser473, and the phosphorylation and subsequent subcellular localization of forkhead box O1a (FOXO1a), a physiologic substrate of Akt.[21] In contrast, solenopsin did not affect the activity of purified PI3K or PDK1, which is the kinase that phosphorylates Akt at Thr308. Taken together, our results imply that solenopsin exerts its effects on Akt activity in cells by inhibiting a step in the signaling pathway that lies upstream of PI3K.

Materials and methods

Synthesis of solenopsin and solenopsin derivatives

Solenopsin analogs were synthesized beginning with 4-chloropyridine and alkyl magnesium halides, resulting in a 2-alkyl-4-chloro tetrahydropyridine, which

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was further modified by lithiation and reduction with a hydrogen and palladium/carbon catalyst.[22] Derivatives S1, S2, and S3 were prepared this way from commercially available alkyl Grignard reagents (Sigma-Aldrich, St Louis, MO). Other reagents were synthesized according to the method of Beak and Lee,[23] in which N-Boc–substituted piperidine is lithiated and methylated at the 6 position with dimethylsulfate, followed by addition of a formyl group at the 2′ position. The 2′ position was modified by a Wittig reaction, generating 2′alkenyl–substituted 6-methylpiperidines, which were subsequently hydrogenated to generate derivatives S5 to S19. Derivatives were converted to hydrochloride salts through passage of hydrogen chloride through solutions of the solenopsin derivatives.

Other reagents were synthesized by lithiating N-Boc–substituted piperidine and methylating it at the 6 position with dimethylsulfate, followed by addition of a formyl group at the 2′ position.[23,24] The 2′ position was modified by a Wittig reaction, generating 2′alkenyl substituted 6-methylpiperidines, which were subsequently hydrogenated to generate derivatives S5 to S19. Derivatives were converted to hydrochloride salts through passage of hydrogen chloride through solutions of the solenopsin derivatives.

Structure activity relationship in SVR angiogenesis assay

SVR cells are murine endothelial cells immortalized by infection with an ecotropic retrovirus encoding SV40 large T antigen, followed by transformation with oncogenic H-ras, and have been used extensively as a screen for angiogenesis inhibitors.[16-18,25] SVR cells were plated at a concentration of 10 000 cells/well, as previously described,[18] and treated with solenopsin or solenopsin analogs (Figure 1) at concentrations varying from 0 to 6 μg/mL for 48 hours, at which point cells were counted with a Coulter Counter (Coulter, Hialeah, FL).

Kinase inhibition assays

Solenopsin and its analogs were tested in vitro for inhibitory activity against the following enzymes as previously described[26]: MKK1, ERK2, JNK1, p38α MAPK, p38β MAPK, p38γ MAPK, p38δ MAPK, RSK1, MAPKAP-K2, MSK1, PRAK, PKA, PKCα, PDK1, AKT1, SGK, S6K1, GSK3β, ROCK-II, AMPK, CHK1, CK2, PHK, LCK, CSK, CDK2/cyclin A, CK1, DYRK1a, and PP2a as previously performed for honokiol.[18] Solenopsin and its analogs were tested against purified recombinant PI3K p110alpha/p85alpha complex measuring the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3).[27]

Western blot analysis

Cells were grown on 10-cm dishes and allowed to reach approximately 80% confluence before protein isolation. Sample aliquots normalized for protein quantities were size fractionated by SDS-PAGE, and the proteins were transferred to a PVDF membrane. The blots were incubated in blocking solution; PBS with 5% (wt/vol) powdered nonfat milk for 1 hour at room temperature (RT). The blots were then incubated overnight in sheep polyclonal anti-Akt (Upstate Biotechnology, Charlottesville, VA) or rabbit polyclonal antiphosphorylated Akt (Ser 473; Cell Signaling Technology, Danvers, MA). Rabbit polyclonal antibodies against insulin receptor substrate 1 (IRS1) were from Upstate Biotechnology, and mouse monoclonal antiphosphotyrosine (P-Tyr-100) was from Cell Signaling Technology. Protein A–agarose beads and secondary antibodies conjugated to horseradish peroxidase (HRP) were from Santa Cruz Biotechnology (Santa Cruz, CA). Dulbecco modified Eagle medium (DMEM) was from Hyclone (Logan, UT), and serum was from Valley Biomedical (Winchester, VA). All chemicals, unless otherwise mentioned, were obtained from Sigma-Aldrich.

FOXO1a and RevGFP export assay

The FOXO1a export assay is described in Kau et al.[25] Briefly, 786-O cells were plated onto black, 384-well, clear-bottom plates (Costar, Corning, NY) in 50 μL DMEM/5% fetal clone and infected with Ad-FKHR adenovirus. For compound treatment, solenopsin A was serially diluted starting from 80 μM in a separate 384-well plate before transfer onto infected cells. Cells were incubated with compound for 1 hour before fixation with 3.7% formaldehyde and then stained with M5 anti-FLAG antibody (Sigma-Aldrich), Alexa Fluor 594 goat antimouse antibody (Invitrogen, Carlsbad, CA) and Hoechst 33258 (Sigma-Aldrich).

RevGFP export inhibition is also described in Kau et al.[25] U2OS-RevGFP cells were seeded onto clear-bottom, black, 384-well plates in 50 μL complete media. Cells were allowed to attach and grow overnight before treatment with solenopsin A. Solenopsin A dilutions were made as described in the FOXO1a export assay. Cells were incubated with compound for 1 hour before fixation with 3.7% formaldehyde and nuclei staining with Hoechst 33258.

Zebrafish stocks

Zebrafish were grown and maintained at 28.5°C. Matings were routinely carried out at 28.5°C, and the embryos were staged according to established protocols.[28] Embryos were staged according to time after fertilization and morphology. Transgenic Fli-EGFP fish (*TG(fli1:EGFP)*^{y1})[29] were purchased from the Zebrafish Information Network (ZFIN, Eugene, OR).

Drug studies

A number of embryos (20-40) from a transgenic mating pair were collected and incubated at 28.5°C with 2.0 mL of egg water in a 6-well plate. At 6 hours after fertilization, egg water was replaced with fresh egg water (2.0 mL) containing drugs (solenopsins A and S3) at concentrations (3.75, 5, and 6 μg/mL) determined from a preliminary dose curve study. DMSO controls were also included each time an experiment was performed. Phenylthiourea (PTU) was added to a final concentration of 0.003% to prevent pigmentation at 10 to 12 hours after fertilization. Embryos were allowed to develop to the required stages at 28.5°C until ready for confocal microscopy. Fish were placed onto glass coverslips embedded into 35-mm dishes (MatTek Corp, Ashland, MA), anesthetized with tricaine (0.016%), and examined with a Zeiss Axiovert 100M microscope using 10× 10.3 NA and 25× 10.3 NA objectives (Zeiss, Thornwood, NY). Images were generated using a Zeiss LSM 510 laser-scanning microscope and were processed using Zeiss Aim software. These experiments were performed in triplicate. Confocal images were captured at the Cell and Cancer Biology Branch Confocal Microscopy Core facility (National Cancer Institutes, National Institutes of Health).

Studies of the effect of solenopsin on cells

3T3-L1 cells were serum-starved in DMEM + 0.2% BSA for 2 hours and treated with 30 μM solenopsin A for 20 minutes prior to stimulating with 1 μM insulin for 10 minutes. Plates were washed 3 times with ice-cold PBS. Cells were scraped into lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 4 mM Na_3VO_4, 200 mM NaF, 20 mM $Na_4P_2O_7$, 10 mM EDTA, 10% glycerol, 1% Triton X-100, and 1:100 protease inhibitor cocktail), and the assay was performed on the cell lysates as mentioned previously.[30] Solenopsin (5 nM-100 μM) was tested in NIH3T3 cells for its ability to inhibit PDGF-induced phospho-Akt formation. Phospho-Akt was determined using the LI-COR Odyssey (Lincoln, NE) in-cell Western protocol.[31]

Results

The SVR proliferation assay is a broad screen that examines the ability of compounds to inhibit the growth of ras-transformed endothelial cells by inhibiting either ras$^-$-specific[17,25] or endothelial-specific[18] signaling. Compounds that inhibit SVR cells are tested routinely on nonendothelial cells to determine whether or not the inhibition is endothelial specific. Solenopsin and a series of related tetrahydropyridines (Figure 1) were tested as angiogenesis inhibitors using the SVR endothelial cell proliferation assay; among all the closely related solenopsin analogs, only solenopsin A significantly impaired SVR proliferation (Figure 2). We thus chose solenopsin for further mechanistic studies.

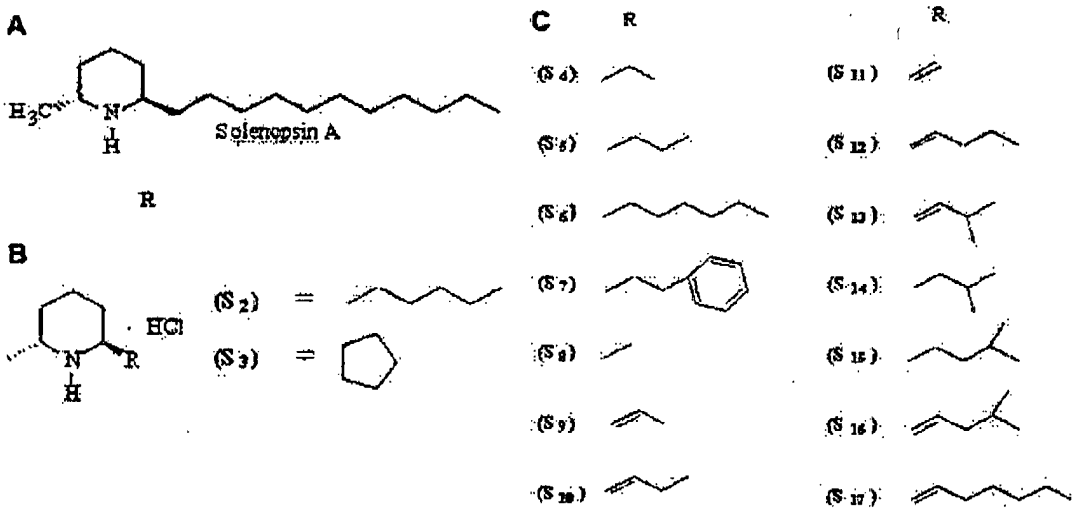

Figure 1. Structure of solenopsin and solenopsin analogs.

Inhibition of kinases

Solenopsin was tested against a panel of 28 kinases and 1 protein phosphatase in vitro (Table 1). These experiments revealed that solenopsin at 10 μM inhibited Akt by 50%. Apart from ribosomal protein S6 kinase 1 (RSK1), which was inhibited to a similar extent, no other enzyme in the panel was inhibited significantly. The inhibition of Akt by solenopsin was found to be competitive with respect to ATP, as inhibition of Akt increased with decreasing concentrations of ATP in the assay (Table 2). Solenopsin did not inhibit PDK1 (Table 1), an upstream activator of Akt, nor did it inhibit the purified recombinant PI3K p110alpha/p85alpha complex in a cell-free assay measuring the conversion of PIP2 to PIP3.

Solenopsin antagonizes Akt function in cell-based assays

Given that solenopsin inhibited Akt in vitro, we wanted to examine its ability to inhibit Akt in cells. Akt is a serine-threonine kinase that phosphorylates multiple substrates, including members of the forkhead family of transcription factors (FKHD/FOXO). Once these FOXO proteins are phosphorylated, they are transported from the nucleus to the cytoplasm. Recently, Kau et al described a high-throughput assay using FLAG-epitope–tagged FOXO1a in

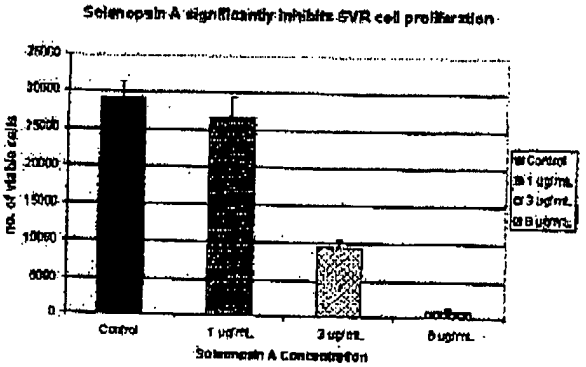

Figure 2. Structure-activity relationship of solenopsin and analogs in SVR angiogenesis assay. Solenopsin and its analogs 1 to 17 were tested for their ability to inhibit the proliferation of SVR endothelial cells at 1 μg/mL, 3 μg/mL, and 8 μg/mL. Only solenopsin A exhibited dose-dependent inhibition of SVR proliferation ($P < .05$). Bars represent average of duplicate experiments, each performed in triplicate. Analog data not shown for brevity.

Table 1. Effect of solenopsin on the activities of protein kinases

Protein kinase	Activity, % control ± SD
MKK1	110 ± 1
ERK2	93 ± 3
JNK1	105 ± 2
p38α MAPK	82 ± 3
p38β2 MAPK	96 ± 2
p38γ MAPK	91 ± 2
p38δ MAPK	100 ± 6
RSK1	46 ± 1
MAPKAP-K2	93 ± 6
MSK1	86 ± 4
PRAK	121 ± 6
PKA	137 ± 4
PKCα	99 ± 9
PDK1	136 ± 6
AKT1	51 ± 6
SGK	87 ± 6
SGK1	81 ± 13
GSK3β	108 ± 2
ROCK-II	104 ± 1
AMPK	98 ± 7
CHK1	84 ± 5
CK2	116 ± 1
PHK	105 ± 0
LCK	89 ± 0
CSK	101 ± 3
CDK2/cyclin A	95 ± 1
CK1	109 ± 4
DYRK1a	86 ± 5
PP2a	114 ± 6

Each protein kinase was assayed in duplicate at 0.1 mM ATP in the presence and absence of 10 μM solenopsin. The results are presented as the percentage of activity remaining in the presence of solenopsin (average of duplicate determinations). Similar results were obtained in another independent experiment. Each protein was expressed, purified, and assayed as described previously.[30,31] MKK indicates MAPK kinase; ERK, extracellular signal-related kinase; JNK, c-Jun N-terminal kinase; RSK, p90 ribosomal S6 kinase; MAPKAP-K2, MAPK-activated protein kinase 2; MSK, mitogen- and stress-activated kinase; PRAK, p38-regulated/activated kinase; PKA, cAMP-dependent protein kinase; PKC protein kinase C; PDK1, 3-phosphoinositide–dependent protein kinase 1; AKT1, protein kinase Bα; SGK, serum and glucocorticoid-induced protein kinase; S6K, p70 ribosomal protein S6 kinase; GSK3, glycogen synthase kinase-3; ROCK, Rho-dependent kinase; AMPK, AMP-activated protein kinase; CHK1, checkpoint kinase 1; CK2, casein kinase 2; PHK, phosphorylase kinase; LCK, lymphocyte kinase; CSK; C-terminal Src kinase; CDK2, cyclin-dependent kinase 2-cyclin A complex; DYRK, dual specificity tyrosine phosphorylation regulated kinase; and PP2a, protein phosphatase 2A.

Table 2. Solenopsin inhibition of PKB/Akt is competitive with ATP

ATP concentration, μM	Activity, % control ± SD
1	25.6 ± 9.6
5	48.4 ± 3.6
20	64.3 ± 5.5
100	75.4 ± 7.3

Solenopsin A (10 mM) was tested for its ability to inhibit PKBα at a range of ATP concentrations. The results are presented as the percentage of activity remaining in the presence of solenopsin relative to the activity measured in the absence of solenopsin. Results are expressed as the average of duplicate determinations. Similar results were obtained in several other experiments.

cells null in PTEN to rapidly assess the ability of compounds to inhibit nuclear export of FOXO proteins.[25] Consistent with its inhibitory activity on Akt, solenopsin A inhibited the nuclear export of FOXO1a. This inhibition was specific, as export of RevGFP, a protein whose nuclear export does not depend on Akt, was not inhibited by solenopsin A (Figure 3).

Solenopsin inhibits insulin-mediated PI3K activation

Treatment of cells with solenopsin did not inhibit insulin-stimulated tyrosine phosphorylation of IRS1 (Figure 4A), but blocked insulin-induced, PI3K-dependent generation of 3-phosphoinositides (Figure 4B). Consistent with inhibition in cells occurring at a level between IRS1 and PI3K, solenopsin also inhibited signaling downstream of PI3K, namely the phosphorylation of Akt at Thr308 (Figure 5) and Ser473 (Figures 4C, 5), which are catalyzed by PDK1 and the mTORC2, respectively, and the phosphorylation of FOXO1A at Ser256 (Figure 5). In contrast, solenopsin did not inhibit PDGF-induced phosphorylation of Akt in NIH3T3 cells except at cytotoxic doses (data not shown), suggesting that solenopsin's activity may be specific to insulin signaling or pathway and/or cell dependent.

Solenopsin A inhibits embryonic angiogenesis in zebrafish

Given that solenopsin A inhibited angiogenesis in vitro, we used a zebrafish model system to determine whether it could suppress angiogenesis in vivo. Zebrafish embryos are transparent, and drugs dissolved in DMSO are readily permeable through the chorion. Solenopsin A and an inactive solenopsin analog (S3) were incubated with embryos from a transgenic *(TG(fli1:EGFP)^{y1})*[29] zebrafish line that carries a 15-kb promoter of friend leukemia integration-1 transcription factor (fli-1), which drives GFP expression in the endothelium. Treatment with solenopsin, but not the

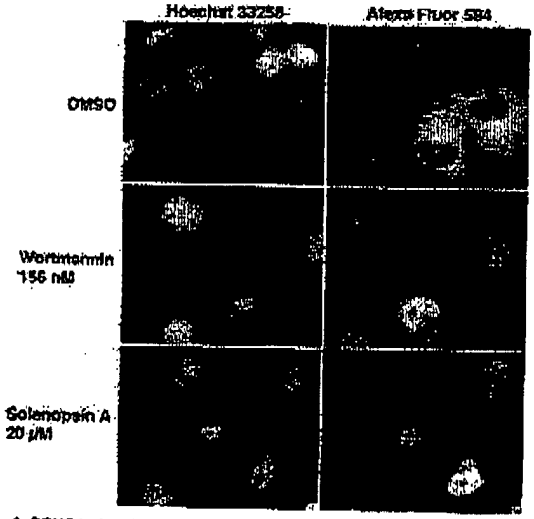

Figure 3. FOXO1a localization after treatment with solenopsin. 786-O PTEN−/− cells were infected with AdFKHR and treated with DMSO, wortmannin, or solenopsin. Treatment with the negative control, DMSO, resulted in FOXO1a in the cytoplasm, while treatment with the PI3K inhibitor wortmannin relocalized FOXO1a to the nucleus. Similarly, solenopsin also relocalized FOXO1a to the nucleus. Wortmannin is used as a positive control.

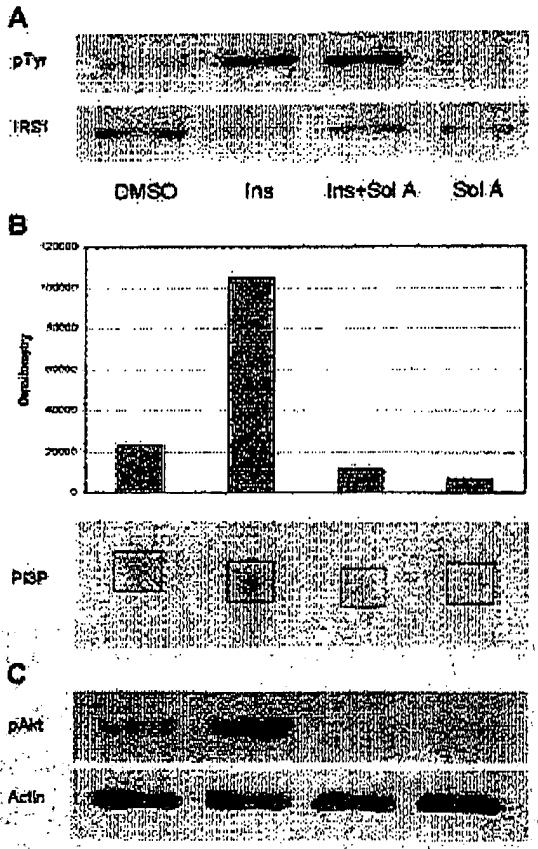

Figure 4. Solenopsin inhibits insulin-stimulated PI3K signaling in 3T3-L1 cells. 3T3-L1 fibroblasts were treated with 30 μM solenopsin A for 20 minutes prior to stimulating with 1 μM insulin for 10 minutes. (A) Insulin-stimulated, IRS-1–associated PI3K activity was assessed by measuring the incorporation of labeled phosphate from [γ^{32}P]ATP into phosphatidylinositol. Labeled phosphatidylinositol 3-phosphate was resolved from ATP by thin-layer chromatography, which was visualized using a Storm PhosphorImager. Intensity of the bands was quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). (B) Cell lysates were resolved by SDS-PAGE, Western-blotted with anti–phospho-Akt (Ser473) antibodies, and detected by enhanced chemiluminescence. (C) IRS-1 was immunoprecipitated from cell lysates as in panel A, but the resulting immunoprecipitates were then resolved by SDS-PAGE and immunoblotted with antiphosphotyrosine or anti–IRS-1 antibodies.

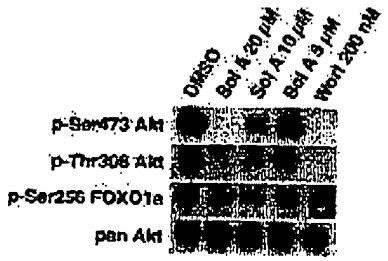

Figure 5. Phospho-Akt and phospho-FOXO1a immunoblot. AdFKHR-infected 786-O cells were treated with DMSO, wortmannin, or solenopsin in decreasing concentrations. Solenopsin, like wortmannin, reduces phospho-Ser473 Akt, phospho-Thr308 Akt, and phospho-Ser256 FOXO1a levels.

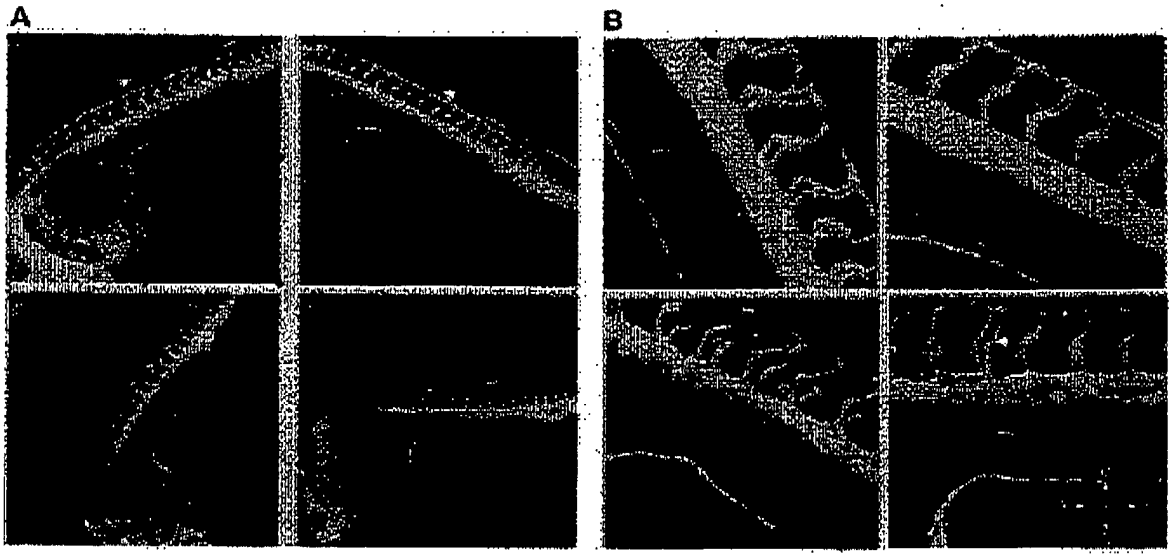

Figure 6. Effect of solenopsin on vascular development in zebrafish. Confocal images of drug-treated embryos shown here are primarily lateral or dorsolateral views of the trunk vasculature of different *TG(fli1:EGFP)*[y1] embryos at 32 hours after fertilization. In both panels (A, 10 ×; B, 25 ×), i and iii represent solenopsin 3–treated embryos while ii and iv represent solenopsin A–treated embryos at 5 and 6 μg/mL, respectively. (A) In i and iii, the primary sprouts from the dorsal aorta and posterior cardinal vein have split at the level of the dorsolateral surface of the neural tube, and branches from adjacent primary sprouts are interconnected to form the paired longitudinal anastomotic vessels (DLAV, white arrowhead). In ii and iv, the primary sprouts (red arrowhead) have delayed considerably in reaching the dorsolateral surface of the neural tube. (B) In high power (25 ×), the absence of DLAV is denoted by an arrow (iv), and the spacing between the sprouts (white arrowhead in iv; red arrowhead in ii) is distinct.

inactive solenopsin analog S3, delayed intersomitic vessel sprouts arising from the dorsal aorta (Figure 6). These effects differ from those reported previously for VEGFR2 inhibitors in zebrafish development[33] in which they injected dominant active Akt constructs that rescued VEGFR2's intersomitic vessel defect when tested at 24 hours after fertilization. Here, we tested the effect of solenopsin at 6 hours after fertilization. The vasculogenic vessels such as the dorsal aorta and the posterior cardinal vein formed appropriately in solenopsin-treated embryos, suggesting that solenopsin may delay angiogenic precursors or sprouts from reaching their target. A potential reason for these differences is that VEGFR2 inhibitors may affect the development of tissues that express VEGFR2, while Akt inhibitors such as solenopsin may affect nonvascular tissues, leading to the different phenotype.

Discussion

Solenopsis invicta, the fire ant, is a major pest in the United States, infesting more than 290 million acres. The ant is capable of multiple stings, and secretes venom that consists of the alkaloid solenopsin and venom proteins.[34-36] Repeated stings can cause death to animals and humans through a direct action of the alkaloid or allergic reaction to the protein. The mechanism of action of solenopsin has not previously been determined. Solenopsin and solenopsin analogs were initially synthesized in an effort to inhibit production of solenopsin by a feedback mechanism in fire ants.

We tested solenopsin and solenopsin analogs in the SVR angiogenesis assay, which measures the ability of compounds to inhibit ras-transformed endothelial cells. Of the compounds tested, only the naturally occurring solenopsin A had activity against SVR cells.

The PI3K signaling pathway is known to play a critical role in angiogenesis; therefore, we investigated whether solenopsin affected this pathway. Interestingly, we found that solenopsin did not affect insulin-induced tyrosine phosphorylation of IRS1, but suppressed the activation of PI3K and hence the phosphorylation events that lie downstream of PI3K, such as the insulin-induced phosphorylation of Akt at Thr308 and Ser473 and the phosphorylation of FOXO1A, a physiologic substrate of Akt. However, we also found that solenopsin did not inhibit purified PI3K or PDK1 (the protein kinase which phosphorylates Akt at Thr308) in vitro. Taken together, our results suggest that solenopsin blocks the signaling pathway downstream of IRS1 but upstream of PI3K, perhaps by disrupting the interaction between IRS1 and the p85 regulatory subunit of PI3K or by altering the location of IGFR in lipid rafts.

Interestingly, we also found that solenopsin inhibits Akt in vitro, and that the inhibition was relatively selective, since only 1 other protein kinase (RSK1) of 28 other kinases tested was inhibited. However, the inhibition was competitive with respect to ATP, and the IC_{50} value determined at 0.1 mM ATP was 5 to 10 μM (Table 2). It is therefore unclear whether Akt would be inhibited significantly in cells at the concentrations used in this study (20-30 μM), since the intracellular concentration of ATP is in the millimolar range. Nevertheless, the relatively selective inhibition of Akt by solenopsin in vitro is of interest because relatively few inhibitors of Akt have been developed, and Akt is a prominent pharmacologic target in cancer and inflammatory disorders.

Phospholipid ethers have been demonstrated to have activity against Akt, as well as potential alternative targets.[15] Solenopsin shares the long alkyl side chains seen in phospholipid ethers and resembles miltefosine and perifosine by having a positively charged amine group and alkyl chain. Miltefosine has been shown to have antileishmanial activity in humans and to cause insulin resistance in muscle.[37] Perifosine is in clinical trials as a PI3K/Akt inhibitor in advanced cancer.[38,39]

Solenopsin and its derivatives are amenable to large-scale synthesis, and because of its free secondary amine structure solenopsin can be readily conjugated to other molecules for targeted delivery. Recently, advances have been made in the identification of molecules that are specific for tumors and tumor endothelium. Conjugation of solenopsin to these molecules may provide a novel and specific therapy for advanced neoplasms, both in terms of treatment of patients directly and purging of autologous bone marrow for transplantation.[40,41]

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Authorship

Contribution: T.K., M.K., K.N., R.R., K.Y., W.M., A.F., J.C., J.B., P.C., D.W., S.F., and B.G. performed experiments; J.L.A., S.A.S., C.J.V., P.A.S., and J.P.B. analyzed data; and J.L.A., B.N.P., and P.C. wrote the manuscript.

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Solenopsin A, a Venom Alkaloid from the Fire Ant *Solenopsis invicta*, Inhibits Quorum-Sensing Signaling in *Pseudomonas aeruginosa*

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In *Pseudomonas aeruginosa*, quorum-sensing (QS) signaling regulates the expression of virulence factors and thus represents an attractive new target for anti-infective therapy. In the present study, we investigated whether solenopsin A, a venom alkaloid from the fire ant, possessed agonistic or antagonistic QS signaling activity in *P. aeruginosa*. We evaluated the modulation of virulence factor expression and transcriptional levels of QS-regulated genes in *P. aeruginosa* by solenopsin A and demonstrated that solenopsin A efficiently disrupted QS signaling. Interestingly, exogenously added C_4–homoserine lactone (HSL), but not 3-oxo-C_{12}-HSL, restored *P. aeruginosa* QS signaling, suggesting that solenopsin A targets the C_4-HSL–dependent *rhl* QS system.

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen that is able to infect insects, plants, and humans [1]. Importantly, chronic *P. aeruginosa* infections are common in patients with cystic fibrosis, causing serious medical complications. The virulence of *P. aeruginosa* is regulated in a population density–dependent manner, a microbial process known as "quorum sensing" (QS). QS is a means of intercellular communication used by both gram-negative and gram-positive bacteria and is mediated by small, diffusible molecules termed "autoinducers" [2]. QS allows bacteria to control gene expression in a cell density–dependent manner, and, notably, genes regulated by QS encode a range of virulence factors, such as toxins and proteins involved in biofilm formation. Therefore, QS signaling systems might represent attractive new targets for antimicrobial therapy [3]. In fact, a number of studies aiming to control bacterial virulence via disruption of QS signaling using synthetic small molecules, natural products, or antibodies have been conducted [3–6].

P. aeruginosa possesses 2 LuxR/I-type QS systems, termed "*las*" and "*rhl*," which use 2 distinct acyl-homoserine lactone (AHL) autoinducers, 3-oxo-C_{12}-HSL and C_4-HSL, respectively. The *las* QS signaling system has been shown not only to regulate the production of virulence factors but also to influence the *rhl* QS circuit [1]. However, it has been demonstrated that the *rhl* QS system itself plays a significant role in controlling the pathogenicity of *P. aeruginosa*, for example, through the production of pyocyanin and elastase B and the formation of biofilms. Most inhibitors targeting the *P. aeruginosa* QS signaling circuits reported to date have been structural mimics of its autoinducers [4]. Recently, a new immunotherapeutic approach has been reported that uses QS-quenching anti-AHL antibodies [6].

Solenopsin A (*trans*-2-methyl-6-undecylpiperidine; solenopsin), a venom alkaloid from the fire ant *Solenopsis invicta*, exhibits global structural similarity to 3-oxo-C_{12}-HSL, possessing a long hydrocarbon chain attached to piperidine, a nitrogen-containing heterocycle, via a chiral carbon (figure 1) [7]. In addition, it has been shown that both solenopsin and 3-oxo-C_{12}-HSL possess antimicrobial activity against gram-positive bacteria [8, 9]. Moreover, a recent study demonstrated that solenopsin and 3-oxo-C_{12}-HSL exert a wide range of biochemical effects on mammalian cells [7, 10]. Thus, in the present study, we investigated solenopsin for its potential to act as a QS signaling agonist or antagonist in *P. aeruginosa*. We demonstrate here that it efficiently suppresses QS signaling, resulting in a decrease in virulent factor production and biofilm formation. More interestingly, in contrast to our initial expectation, our data suggest that the main inhibitory effect of solenopsin might be exerted on *rhl* signaling.

Methods. PAO1-I, PAO1-O, and PDO 100 strains were provided by B. Iglewski (University of Rochester). Syntheses of solenopsin and its analogues have been described elsewhere [7]. Virulence factor assays, including biofilm analysis, were per-

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Potential conflicts of interest: J.L.A. and J.P.B. have filed for US and international patents for solenopsin.

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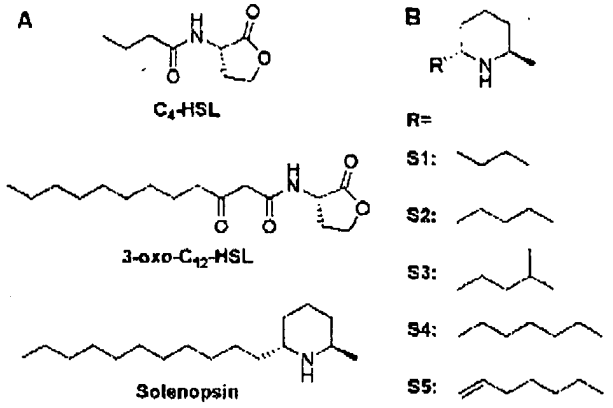

Figure 1. Structures of C_4–homoserine lactone (HSL), 3-oxo-C_{12}-HSL, solenopsin A, and solenopsin analogues (S1–S5).

formed following methods using PAO1-I, PAO1-O, or PDO 100 that have been described elsewhere [6]. For real-time polymerase chain reaction (PCR) analysis, fresh Luria-Bertani (LB) medium was inoculated with overnight-grown PAO1 (optical density $[OD]_{600}$, ~0.05), and cells were grown for ~3 h to an OD_{600} of ~ 0.5. Cells were diluted again into fresh LB containing solenopsin (50 μmol/L) and grown for ~5 h to an OD_{600} of ~0.6 and for ~6 h to an OD_{600} of ~2.2 for log- and stationary-growing phases, respectively. Cells were collected by centrifugation, total RNA was purified, and real-time PCR analysis was conducted as described elsewhere [5]. Nucleotide sequences of the primers used for real-time PCR analysis are as follows: *rpoD* forward, 5'-GGGCGAAGAAGGAAATGGTC-3'; *rpoD* reverse, 5'-CAGGTGGCGTAGGTGGAGAA-3'; *lasB* forward, 5'-TCATCACCGTCGACATGAACAGCA-3'; *lasB* reverse, 5'-AGTCCCGGTACAGTTTGAACACCA-3'; *rhlI* forward, 5'-TCTCTGAATCGCTGGAAGGGCTTT-3'; *rhlI* reverse, 5'-ATGGTCGAACTGGTCGAATTCCTG-3'; *lasI* forward, 5'-GCTCAAGTGTTCAAGGAGCGCAAA-3'; and *lasI* reverse, 5'-AGTGGTATCGAGAATTCGCCAGCA-3'.

Results and discussion. Pyocyanin, a QS-controlled virulence factor of *P. aeruginosa*, has been shown to cause serious tissue damage in chronic lung infection [11]. To examine whether solenopsin has an effect on AHL-mediated QS signaling in *P. aeruginosa*, pyocyanin production was investigated in *P. aeruginosa* in the presence or absence of solenopsin. To quantify the amount of pyocyanin produced, an extraction was con-

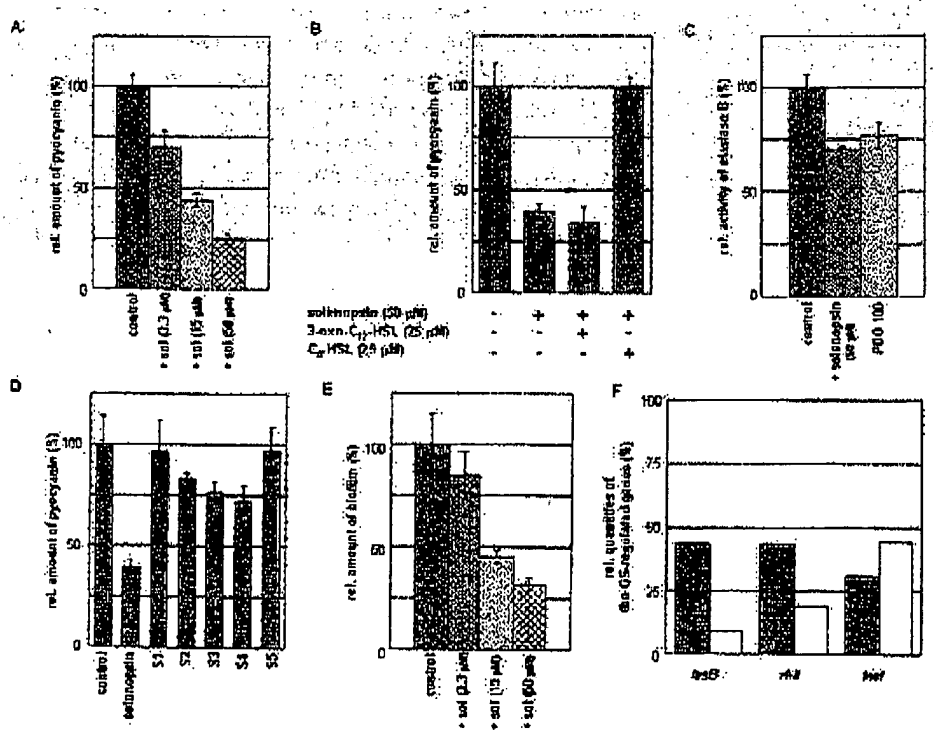

Figure 2. Virulence factor assays and real-time polymerase chain reaction analysis. *A*, Inhibition of pyocyanin production in PAO1-O by solenopsin. *B*, Effects of exogenously added acyl-homoserine lactone on the inhibition of pyocyanin production by solenopsin in PAO1-O. HSL, homoserine lactone. *C*, Elastase B production by PAO1-I in the presence of solenopsin (50 μmol/L) and the Δ*rhlI* strain PDO 100. *D*, Inhibition of pyocyanin production in PAO1-O by solenopsin and its analogues (50 μmol/L). *E*, Inhibition of biofilm formation of PAO1-I by solenopsin. *F*, Analysis of relative transcription levels of the indicated genes in the presence of solenopsin (50 μmol/L). For relative quantification, *rpoD* was used as a reference gene [12]. Gray bars represent the log-growing phase (optical density $[OD]_{600}$, ~0.6), and white bars represent the stationary-growing phase (OD_{600}, ~2.2). QS, quorum sensing.

ducted directly from *P. aeruginosa* growth medium. The absorbance of the acidified aqueous solution containing pyocyanin was measured at 520 nm. As shown in figure 2*A*, a significantly lower amount of pyocyanin (≤50%) was found in the culture grown in the presence of solenopsin, indicating that solenopsin efficiently suppressed QS signaling of *P. aeruginosa* (median effective concentration, ~15 μmol/L). To investigate whether solenopsin acted as an AHL competitor, we tested whether the exogenous addition of the synthetic AHLs could restore pyocyanin production in *P. aeruginosa*. Surprisingly, our data (figure 2*B*) showed that exogenous C_4-HSL, but not 3-oxo-C_{12}-HSL, successfully competed with solenopsin and restored QS signaling in *P. aeruginosa*, suggesting that solenopsin might indeed target the *rhl* signaling system. This is especially noteworthy because there have been only a few reports on small-molecule inhibitors that target the *rhl* system. In light of these results, the modulation of elastase B production was investigated; elastase B is a metalloprotease whose expression is partly controlled by C_4-HSL QS signaling. Figure 2*C* demonstrates that solenopsin decreased elastase B production, although the effect (~30% decrease by 50 μmol/L solenopsin) is not as dramatic as that seen with pyocyanin. We reasoned that elastase B production might be less dependent on *rhl* QS signaling than pyocyanin synthesis. Hence, to support our rationale, we used a Δ*rhlI* mutant strain, PDO 100, in an elastase B assay and showed that 50 μmol/L solenopsin indeed suppressed elastase B production as efficiently as a genetically impaired mutant.

Because solenopsin inhibits the *rhl* QS system in *P. aeruginosa*, it is reasonable to hypothesize that molecules derived from solenopsin containing shorter acyl chains might have an increased antagonistic activity against C_4-HSL. Therefore, several solenopsin analogues (figure 1) prepared previously were investigated [7], but none of the analogues demonstrated increased QS inhibition (figure 2*D*).

As stated above, biofilm formation in *P. aeruginosa* is also regulated by QS signaling. Moreover, a recent study revealed that the *rhlI* mutant showed reduced biofilm formation compared with the wild-type strain, while a *lasI* mutant did not show significant impairment in biofilm formation [13]. Thus, solenopsin was also evaluated for its ability to modulate biofilm formation in *P. aeruginosa* via disruption of *rhl* QS signaling. *P. aeruginosa* was grown under static conditions in a polyvinyl chloride 96-well plate with peptone trypticase soy broth [14]. After 48 h of incubation without agitation, the plate was gently washed, and the biofilm was stained with crystal violet solution for relative quantification. Gratifyingly, our analysis demonstrated that the biofilm formation in *P. aeruginosa* was gradually reduced in the presence of solenopsin in a dose-dependent manner, but no changes were found with the solvent control, again indicating that solenopsin disrupts *rhl* QS signaling in *P. aeruginosa* (figure 2*E*).

Solenopsin has been shown to exert antimicrobial activity against gram-positive bacteria but possessed only weak toxicity toward gram-negative bacteria [8]. To verify that the observed decrease in virulence factor production and biofilm formation was not caused by growth inhibition, we monitored the growth of *P. aeruginosa* in the presence of solenopsin. Notably, no growth defect or retardation was observed over an 8-h period (data not shown), thus strongly suggesting that QS inhibition and the subsequent reduction of virulence factor production is indeed due to competition of solenopsin with C_4-HSL. Last, the transcription of 3 QS-controlled genes—namely, *lasB* (encoding elastase B) as well as *lasI* and *rhlI* (the 2 autoinducer synthase genes)—was analyzed by real-time PCR in the presence or absence of solenopsin [15]. Our data demonstrated that transcription of these QS-controlled genes was decreased ~2-fold (figure 2*F*), further confirming that solenopsin is a QS signaling inhibitor in *P. aeruginosa*.

In summary, we have demonstrated that solenopsin A, a venom alkaloid from the fire ant *S. invicta*, suppressed QS signaling in *P. aeruginosa*, resulting in modulation of virulence factor production as well as biofilm formation. An important implication of our data is that solenopsin might act mainly on the *rhl* QS signaling system as a competitor of C_4-HSL. We have recently introduced a QS-quenching immunotherapeutic strategy that might not be readily applicable to the *rhl* system, because the C_4-HSL molecule might be too diminutive to elicit a specific host immune response against it. In this regard, solenopsin could be a valuable starting point for the discovery of novel C_4-HSL antagonists and thus immensely valuable to the further development of therapeutic *rhl* signaling inhibitors.

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